

# Control of Erythroid Differentiation: Asynchronous Expression of the Anion Transporter and the Peripheral Components of the Membrane Skeleton in AEV- and S13-transformed Cells

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**Abstract.** Chicken erythroblasts transformed with avian erythroblastosis virus or S13 virus provide suitable model systems with which to analyze the maturation of immature erythroblasts into erythrocytes. The transformed cells are blocked in differentiation at around the colony-forming unit-erythroid stage of development but can be induced to differentiate in vitro. Analysis of the expression and assembly of components of the membrane skeleton indicates that these cells simultaneously synthesize  $\alpha$ -spectrin,  $\beta$ -spectrin, ankyrin, and protein 4.1 at levels that are comparable to those of mature erythroblasts. However, they do not express any detectable amounts of anion transporter. The peripheral membrane skeleton components assem-

ble transiently and are subsequently rapidly catabolized, resulting in 20–40-fold lower steady-state levels than are found in maturing erythrocytes. Upon spontaneous or chemically induced terminal differentiation of these cells expression of the anion transporter is initiated with a concomitant increase in the steady-state levels of the peripheral membrane-skeletal components. These results suggest that during erythropoiesis, expression of the peripheral components of the membrane skeleton is initiated earlier than that of the anion transporter. Furthermore, they point a key role for the anion transporter in conferring long-term stability to the assembled erythroid membrane skeleton during terminal differentiation.

ERYTHROPOIESIS in both mammalian and avian species involves a sequence of events whereby a pluripotent stem cell becomes committed to the erythroid cell lineage and thence progresses through at least two replicative progenitor stages, known as burst-forming unit-erythroid (BFU-E)<sup>1</sup> and colony-forming unit-erythroid (CFU-E) stages, before reaching the distinctive erythroblast stages (reviewed in Metcalf and Moore, 1971; Till and McCulloch, 1980). The BFU-E and CFU-E cells have thus far been identified solely in terms of (a) their progeny colony size and hence number of cell divisions from the terminally differentiated erythroid state, (b) their responsiveness to hemopoietic factors such as colony-stimulating factor (interleukin-3) and erythropoietin, and (c) the presence of certain diagnostic surface antigens (Gregory, 1976; Gregory and Eaves, 1978; Samarut and Nigon, 1976; Samarut et al., 1979; Samarut and Bouabdelli, 1980; Till and McCulloch, 1980; Goodman et al., 1985). Part of the terminal differentiation program of these erythroid progenitor cells entails the restructuring of the plasma membrane including the biogenesis of a mem-

brane skeleton, a network of proteins underlying and interacting with the plasma membrane (Branton et al., 1981; Marchesi, 1985). The major component of this network is the heterodimeric protein spectrin, which, in the presence of protein 4.1, interacts with high affinity with short oligomers of actin to form a dense anastomosing network (Branton et al., 1981; Ungewickell et al., 1979; Pinder and Gratzer, 1983; Pinder et al., 1984; Marchesi, 1985). Interaction with the membrane is mediated through the binding of the  $\beta$ -spectrin subunit to ankyrin (Bennett and Stenbuck, 1979a; Luna et al., 1979; Calvert et al., 1980; Litman et al., 1980) which in turn binds to the cytoplasmic domain of the transmembrane anion transporter (Bennett and Stenbuck, 1979b, 1980; Hargreaves et al., 1980). In mammals, glycophorin may provide additional membrane-binding sites through its interaction with protein 4.1 (Anderson and Lovrien, 1984), but as yet an avian equivalent of glycophorin has not been identified.

Synchronized cohorts of proliferative erythroblasts or postmitotic immature erythrocytes can be readily isolated from chick embryos at a stage when they are actively synthesizing and assembling the constituent polypeptides of the membrane skeleton, in sufficient numbers to enable biochemical analysis of their mechanisms of assembly (e.g., Weise and Chan, 1978; Blikstad et al., 1983). Studies with

1. *Abbreviations used in this paper:* AEV, avian erythroblastosis virus; BFU-E, burst-forming unit-erythroid; CFU-E, colony-forming unit-erythroid; MEL, murine erythroleukemia.

these erythroid cells of both the primitive and definitive lineage have indicated that at this stage of erythroid development all members of the erythroid membrane skeleton are expressed simultaneously and accumulate on a per-cell basis by stable assembly (Weise and Chan, 1978; Blikstad et al., 1983; Moon and Lazarides, 1984; Woods and Lazarides, 1985; Staufenbiel and Lazarides, 1986). Hitherto, these studies have not addressed the issue of exactly when in development expression of the components is initiated and whether their expression begins coordinately or asynchronously. Such studies are hindered by the difficulties of identifying erythroid progenitor cells (rather than their daughter colonies) unambiguously and then of isolating them in adequate numbers for biochemical analysis. Avian erythroblastosis virus (AEV)- and S13-transformed erythroid progenitor cells provide a model system for an alternative approach to investigate the mechanisms underlying initiation of the assembly of this membrane domain.

AEVs are replication-defective RNA viruses that cause acute erythroleukemia within 1–2 wk after infection. The target cells in chicken yolk sac and bone marrow appear to be erythroid progenitor cells at the BFU-E stage which then develop to the CFU-E stage before becoming transformed and arrested in development (Gazzolo et al., 1980a, b; Graf et al., 1981; Samarut and Gazzolo, 1982). Transformation abolishes the requirement of these cells for erythropoietin for growth. The AEV ES4 strain is defective in gag and pol sequences which have been replaced by oncogenic erb sequences of cellular origin, v-erb A and v-erb B (Lai et al., 1979; Coffin et al., 1981; Vennström and Bishop, 1982). The v-erb B gene encodes a transmembrane glycoprotein that has tyrosine protein kinase activity and that bears extensive homology with the epidermal growth factor receptor (Hayman et al., 1983; Privalsky et al., 1984; Gilmore et al., 1985; Kris et al., 1985; Yamamoto et al., 1983). The v-erb A gene, which has marked homology with the estrogen receptor (Green, G. L. et al., 1986; Green, S. et al., 1986), is expressed as a gag-erb A fusion protein, localized in the cytoplasm of the transformed cells (Hayman et al., 1979; Bunte et al., 1982). By itself, v-erb A has no transforming activity; rather it appears to potentiate v-erb B activity in addition to reducing the growth requirements of transformed cells and blocking their capacity to differentiate (Graf and Beug, 1978; Graf et al., 1978; Frykberg et al., 1983; Graf and Beug, 1983).

The S13 retrovirus represents a different type of erythroleukemic virus to the AEV group of viruses. Its genome of 8.5 kb encodes a transformation-specific glycoprotein, gp155, that is cleaved posttranslationally into an 85-kD and 70-kD glycoprotein (Benedict et al., 1985; Beug et al., 1985; Hayman et al., 1985). Although the gp70 polypeptide does not appear to bear much structural resemblance to v-erb B, it possesses protein tyrosine kinase activity and in this respect may be functionally similar to the v-erb oncogenic protein encoded by the AEV genome. The S13 virus also transforms cells at a pro-erythroblast stage, inducing rapid proliferation in an erythropoietin-independent manner but, in contrast to AEV, S13 only partially blocks differentiation and does not eliminate the cells' requirement for complex growth media.

We have found that AEV-transformed cells as well as S13-transformed cells express peripheral components of the

erythroid membrane skeleton, namely  $\alpha$ - and  $\beta$ -spectrin, ankyrin, and protein 4.1. However they do not appear to express the anion transporter as determined by immunoblotting, biosynthetic labeling, and Northern analysis. Kinetic analysis has revealed that in AEV-transformed cells, these peripheral components of the membrane skeleton are synthesized in a short labeling period at levels equivalent to the levels found in proliferative primitive erythroid cells. These proteins inefficiently assemble into a membrane skeleton which is then turned over. In contrast, no detectable turnover of newly synthesized assembled protein occurs in maturing primitive erythroblasts. Initiation of spontaneous or chemically induced terminal differentiation of transformed cells is associated with induction of expression of the anion transporter; this is paralleled by an increase in the steady-state levels of the peripheral components of the membrane skeleton. These results indicate that expression of the peripheral components and the anion transporter are independently regulated during erythroid development; initiation of the expression of the peripheral components occurs early in development (by the CFU-E stage) while the expression of the anion transporter is intimately coupled with terminal differentiation. Furthermore, the extent of accumulation of the peripheral skeletal components that occurs during erythroid terminal differentiation is regulated by an increase in the stability of the assembled polypeptides rather than by their transcriptional up regulation. The results point to a key role for the anion transporter in conferring long-term stability to the erythroid membrane skeleton during terminal differentiation.

## Materials and Methods

### Viruses and Cells

The sources of avian erythroblastosis virus strain ES4 and of virus S13 have been described (Lai et al., 1979; Beug et al., 1985; Benedict et al., 1985). Single cell suspensions were prepared by mincing the yolk sacs of 7-d-old chicken embryos and filtering the minced tissue through a narrow mesh, stainless steel screen. The cells were centrifuged at 1,000 rpm for 10 min and then suspended in fresh growth medium at a concentration of  $\sim 2 \times 10^8$  cells/ml. For infection with AEV, growth medium consisted of medium F10 and 10 mM Hepes, pH 7.3,  $5 \times 10^{-5}$  M thioglycerol, and 9% FCS, 8% chicken serum, and 3%  $\text{NaHCO}_3$  (0.33 M). For infection with S13, the growth medium consisted of medium F10 containing 10 mM Hepes, pH 7.3,  $8 \times 10^{-5}$  M mercaptoethanol, 3%  $\text{NaHCO}_3$  (0.33 M), 8% each of fetal calf and chicken serum, 0.08% bovine serum albumin, and 5% water. AEV infection was at a multiplicity of 0.1 for 2 h at 37°C. The AEV-infected cells were then suspended in semi-solid medium consisting of medium F10 with 10 mM Hepes, pH 7.3,  $5 \times 10^{-5}$  thioglycerol, 2%  $\text{NaHCO}_3$  (0.33 M), 8% FCS, 5% chicken serum, 5%  $\text{H}_2\text{O}$ , and 0.9% methocellulose.

Infection with S13 either followed the protocol described above for AEV or was accomplished by seeding the yolk sac cells overnight onto a layer of S13-transformed, x-irradiated chicken embryo fibroblasts. Suspension of S13-infected yolk sac cells was in a medium consisting of medium F10 with 10 mM Hepes, pH 7.3,  $8 \times 10^{-5}$  M mercaptoethanol, 2%  $\text{NaHCO}_3$  (0.33 M), 8% each of FCS and chicken serum, 0.8% BSA, 5%  $\text{H}_2\text{O}$ , and 0.6% methocellulose.

Colonies of transformed cells were picked 14–21 d after infection and were initially plated in the liquid growth media described above. After a given colony had reached cell numbers above  $10^7$ , culture continued with the same liquid growth media except that F10 was replaced by DME. These cells were found to grow best at densities not exceeding  $1 \times 10^6$ , so consequently they were subcultured generally every third day and invariably the day before an experiment. Under these conditions several clones were found to grow up to 4 mo with a doubling time of  $\sim 24$  h. For induction experiments, AEV-transformed cells were transferred to the CFU-E medium de-

scribed by Beug et al. (1982a) containing 1 mM butyrate and 1 U/ml erythropoietin (Amgen, Thousand Oaks, CA).

Mitotic primitive erythroblasts were isolated from 3.5–4-d chick embryos by carefully excising the embryo and surrounding blood islands from the egg, teasing them apart and letting them bleed into MEM supplemented with 2% FCS. The cells were passed through several layers of cheesecloth and then through cotton wool before washing three times in MEM, 2% FCS. The cells were then incubated for 30 min in methionine-free medium before labeling experiments.

### Labeling Experiments

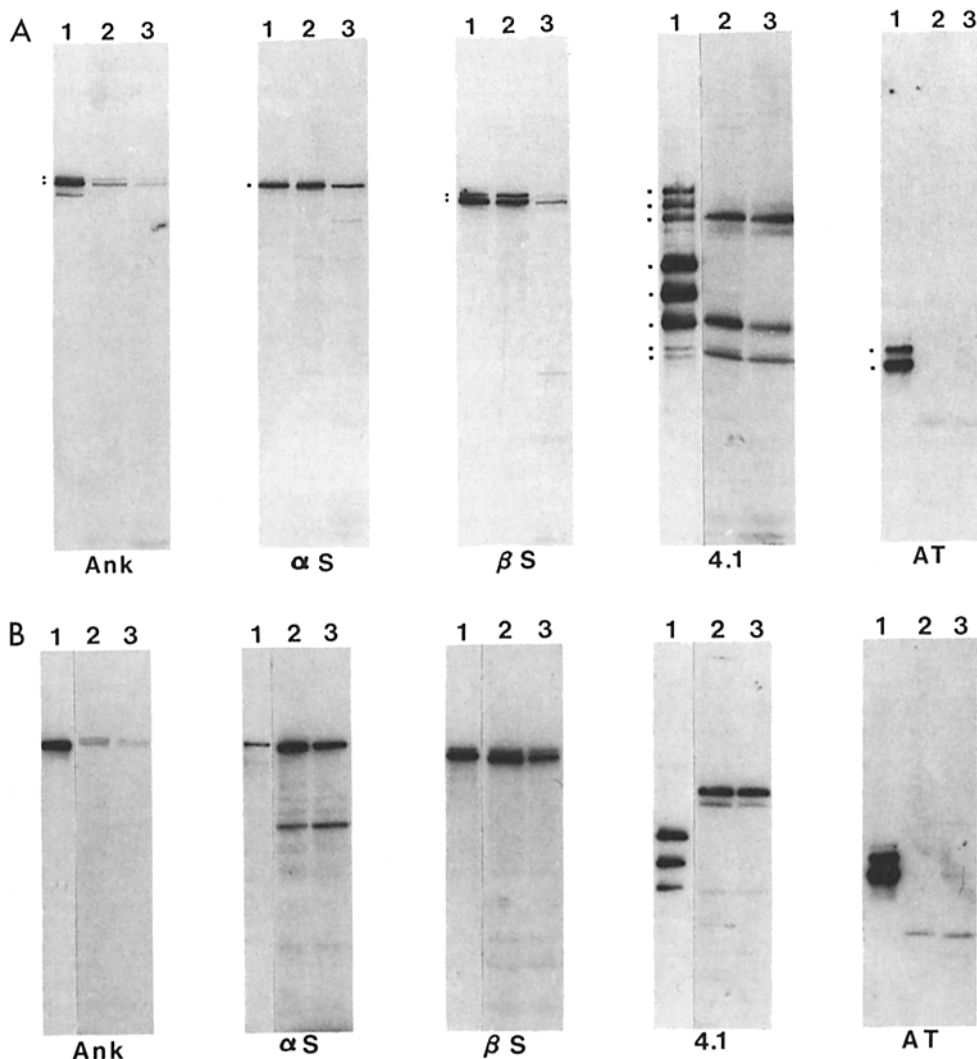
AEV cells were washed three times in methionine-free DME before suspending in labeling medium (methionine-free DME, 10% dialyzed FCS,  $5 \times 10^{-5}$  M thioglycerol) to preincubate for 20 min at 37.5°C in a humidified atmosphere containing 5% CO<sub>2</sub>. For pulse-chase experiments,  $7 \times 10^7$  cells were suspended in 5 ml of labeling medium containing 600  $\mu$ Ci [<sup>35</sup>S]methionine and incubated for 8 min before pelleting the cells (total labeling time 10 min). Cells were resuspended in normal growth medium containing 2 mM unlabeled methionine and divided into aliquots of  $10^7$  cells for further incubation. The aliquots of cells were sampled at appropriate intervals of chase time and added to excess ice-cold medium for processing. For continuous pulse experiments aliquots of cells were labeled with 200  $\mu$ Ci [<sup>35</sup>S]methionine at a starting density of  $10^6$  cells/ml in labeling medium supplemented with 2  $\mu$ M methionine in order to prevent methionine deprivation. The cells were lysed sequentially in identical volumes of hypotonic (10 mM Tris-HCl, pH 7.4, 5 mM MgCl<sub>2</sub>, 2 mM EGTA, 2 mM phenylmethylsulfonyl fluoride [PMSF], and 1 mM aprotinin) and 0.5% Triton X-100 lysis buffer (130 mM NaCl, 10 mM Tris-HCl, 5 mM MgCl<sub>2</sub>,

2 mM EGTA, 0.5% Triton X-100, 2 mM PMSF, 1 mM aprotinin), taking care to resuspend ghosts with a minimum of agitation. The final pellet was resuspended in an equal volume of hypotonic buffer. All these operations were carried out at 4°C. This yielded an operationally defined cytoplasmic (hypotonic lysate), noncross-linked membrane-associated (Triton lysate) and insoluble (cytoskeletal plus nuclear) fraction respectively, as described previously (Woods and Lazarides, 1985). Samples were denatured by addition of 1/5 vol 5% SDS, 5 mM EGTA, 10 mM Tris-HCl, pH 7.4, 2 mM  $\beta$ -mercaptoethanol followed by sonication for the insoluble fractions.

Each lysate for each time point was divided into equal aliquots, pre-cleared with preimmune serum before immunoprecipitation with anti- $\alpha$ -spectrin (Repasky et al., 1982), anti- $\beta$ -spectrin (Nelson and Lazarides, 1984), anti-ankyrin (Nelson and Lazarides, 1984), anti-protein 4.1 (Granger and Lazarides, 1984a) and anti-anion transporter (Cox et al., 1985) specific antibodies. Immunoprecipitation, autoradiography, and quantitation of labeled protein was carried out as described previously (Woods and Lazarides, 1985).

### Immunoblotting

Cells were washed twice in DME and then solubilized either directly in SDS sample buffer or after making hypotonic, 0.5% Triton X-100 and insoluble fractions to give the equivalent of  $10^6$  cells per 25  $\mu$ l of final SDS sample. Samples were subjected to 7.5% SDS PAGE and transferred to nitrocellulose. Filters were probed with one of the antibodies mentioned above or anti-vimentin (Granger and Lazarides, 1982) at a dilution of 1:1,000, washed and probed with <sup>125</sup>I-protein A (Granger and Lazarides, 1984a). After exposure to Kodak XAR-5 film, labeled bands were cut out and counted on a gamma counter. Quantitative results were ensured by using cell loadings



**Figure 1.** AEV- and S13-transformed erythroid cells express the peripheral components of the membrane skeleton but not the anion transporter. Immunoblots of  $3 \times 10^5$  mature chicken erythrocyte ghosts (lanes 1) or the Triton X-100-insoluble (lanes 2) and -soluble (lanes 3) fractions from  $3 \times 10^6$  AEV-cells (A) or S13 cells (B). Ank, ankyrin;  $\alpha$ S,  $\alpha$ -spectrin;  $\beta$ S,  $\beta$ -spectrin; 4.1, protein 4.1; AT, anion transporter. Antigens designated by dots. The differences in the relative amounts of the 4.1 variants between Fig. 1 A (AEV) and 1 B (S13) are due to differences in exposure time.

within the range that gave linear increases in signal ( $1-6 \times 10^6$  cells/lane) and by reducing the number of mature chick erythrocyte cells loaded as standard to give a signal close to that obtained with transformed cells.

## RNA Isolation and Analysis

Cytoplasmic RNA was isolated using the phenol method described in Moon et al. (1985) from either  $10^9$  erythroblasts from 9-d embryos or  $5 \times 10^8$  AEV-transformed cells. Poly(A)<sup>+</sup> RNA was purified through oligo-(dT) cellulose according to the method of Aviv and Leder (1972). Northern analysis was performed as described by Ngai et al. (1984). Essentially RNA was separated on a 1.2% agarose gel containing 6% formaldehyde and transferred to nitrocellulose for 10 h before being baked. Subsequently one set of lanes was probed with a vimentin-specific <sup>32</sup>P-labeled probe (Ngai et al., 1984) and the duplicate set of lanes was probed with a <sup>32</sup>P-labeled probe specific for anion transporter transcripts (Cox et al., 1985).

## Results

### Expression of Peripheral Components of the Erythroid Membrane Skeleton in AEV- and S13-transformed Cells

Steady-state protein analysis carried out by immunoblotting revealed that AEV-transformed cells express  $\alpha$ -spectrin,  $\beta$ -spectrin, ankyrin, and protein 4.1 (Fig. 1 A). A similar result is also observed with S13-transformed cells (Fig. 1 B). Quantitation of immunoblots of AEV cell extracts showed that the steady-state levels of  $\alpha$ -spectrin,  $\beta$ -spectrin, ankyrin, and protein 4.1 were  $\sim 1/5$ ,  $1/24$ ,  $1/29$ , and  $\sim 1/50$ , respectively, of the levels found in mature erythrocytes (Table I). These translate to ratios of  $\beta$ -spectrin to ankyrin to protein 4.1 of  $\sim 1:0.5:0.5$  (Table I) which is similar to the ratios of these proteins found in mitotic primitive erythroblasts (Staufenbiel and Lazarides, 1985). Similar values were found with S13-transformed erythroid cells except that these cells appeared to express even lower levels of ankyrin that translated to a  $\beta$ -spectrin/ankyrin ratio of  $\sim 4:1$  (Table I). The ratio of 5:1 found for  $\alpha$ -spectrin to  $\beta$ -spectrin in both types of transformed cells exceeds the 1:1 stoichiometry observed in developing and mature erythrocytes; this we presume to reflect the concurrent expression of a non-erythroid form of spectrin, a  $\gamma$ -spectrin, but in the absence of a probe for  $\gamma$ -spectrin we could not verify this. Fifteen different AEV-transformed clones were analyzed to check the degree of variation existing between different transformed cell lines. All gave identical quantitative and qualitative steady-state patterns of these proteins (data not shown).

Both the AEV and S13 cells express a similar pattern of variants of protein 4.1, the 155-, 145-, 87-, and 77-kD variants although the upper variants appear to be the more dominant ones in S13-transformed cells (Fig. 1). This pattern differs from the pattern of variants seen in postmitotic erythrocytes; rather they appear to bear a greater similarity to the pattern observed in proliferative primitive or definitive cells (Granger and Lazarides, 1984b; Staufenbiel and Lazarides, 1986) except that in these latter cells the 77-kD variant is more abundant. The same two variants of  $\beta$ -spectrin that exist in immature and mature chick erythrocytes are seen in both types of transformed cells, a major variant of apparent  $M_r$  220,000 and a minor variant with an apparent  $M_r$  of  $\sim 225,000$ . Likewise the pattern of ankyrin variants observed in transformed cells is similar to the pattern observed in mature chick erythrocytes (Fig. 1).

### $\alpha$ -Spectrin $\beta$ -Spectrin, Ankyrin, and Protein 4.1 Are Associated with the Plasma Membrane of Transformed Cells

To establish where the spectrins, ankyrin, and protein 4.1 were localized in transformed cells, we carried out indirect immunofluorescence. All of these proteins appeared to localize at the plasma membrane (data not shown). To examine their localization further, we analyzed their solubility properties. With both types of transformed cells, these proteins cannot be detected at steady state in the hypotonic lysate; rather they appear to be partitioned between a Triton-soluble and cytoskeletal pool, with similar ratios of the four polypeptides within each pool (Table I). When developing and mature erythrocytes are lysed in hypotonic or Triton X-100-containing buffers the spectrins, ankyrin, and protein 4.1 remain quantitatively insoluble at steady state. This is a consequence of their being cross-linked into a skeletal array (Yu et al., 1973; Woods and Lazarides, 1985; Staufenbiel and Lazarides, 1986). Therefore, the solubility properties of these proteins in transformed cells are consistent with the interpretation that these proteins are associated with the plasma membrane but suggest that they are inefficiently cross-linked into a cytoskeleton. No difference in the patterns of  $\beta$ -spectrin and protein 4.1 variants are seen between the Triton-soluble and -insoluble pools (Fig. 1). Ankyrin, however, shows the typical erythrocyte pattern in the insoluble fraction

Table I. Steady-state Ratios of the Peripheral Components of the Membrane Skeleton in Transformed Erythroid Cells

Protein	Adult Erythrocyte/AEV	INSOL/TX	Steady-state ratio of antigen to $\beta$ -spectrin
AEV-transformed erythroid cells			
$\alpha$ -Spectrin	$5.2 \pm 0.6$	1.9	$4.66 \pm 0.24$
$\beta$ -Spectrin	$23.3 \pm 2.8$	4.5	1.0
Ankyrin	$29.0 \pm 3.2$	2.4	$0.49 \pm 0.07$
Protein 4.1	$51.1 \pm 8.2$	1.2	$0.44 \pm 0.09$
Anion transporter	ND	—	—
Vimentin	$5.0 \pm 0.8$	—	—
S13-transformed erythroid cells			
$\alpha$ -Spectrin	4.1	1.2	5.2
$\beta$ -Spectrin	21.6	2.2	1.0
Ankyrin	50.8	2.1	0.22
Protein 4.1	39.4	1.0	0.55
Anion transporter	ND	—	—
Vimentin	3.6	—	—
Induced AEV cells			
$\alpha$ -Spectrin	4.2		3.2
$\beta$ -Spectrin	13.1		1.0
Ankyrin	16.7		0.4
Protein 4.1	26.5		0.5
Anion transporter	10.8		1.2

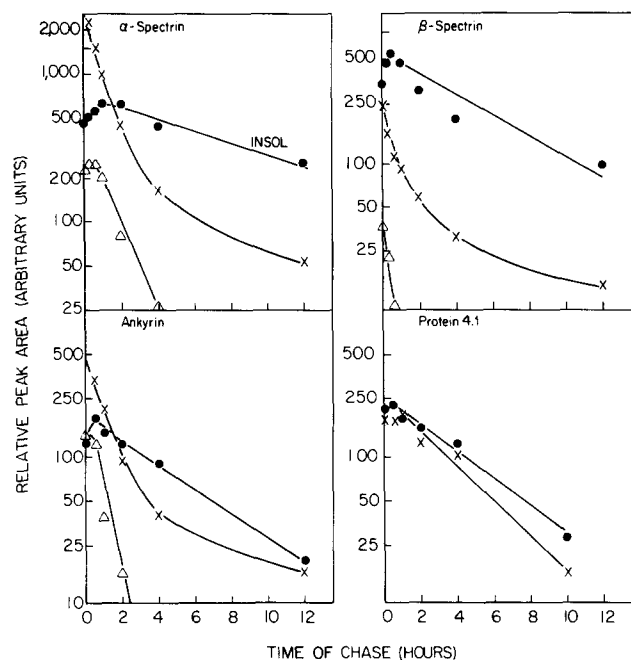
Labeled bands from immunoblots such as the ones shown in Fig. 1 were cut out and counted in a gamma counter. Then the following parameters were calculated: the steady-state ratio of each antigen in a population of transformed erythroid cells compared to mature chick erythrocytes; the steady-state ratio between the Triton-soluble (TX) and -insoluble (INSOL) fractions from transformed cells; and the steady-state ratio of each antigen with respect to  $\beta$ -spectrin existing in transformed cells. The data for AEV-transformed erythroid cells represent the mean of five experiments  $\pm$  SE. The data for S13-transformed erythroid cells and induced AEV cells represent the mean result of two experiments. The data for the induced cells represent the average data for a population of  $3 \times 10^6$  AEV cells out of which 24% are strongly positive for hemoglobin and 23% are weakly positive.

but in the Triton-soluble pool the lower variant ( $\beta$ -goblin of Nelson and Lazarides, 1984) is replaced by a variant of slightly higher apparent molecular weight (Fig. 1). However, since the apparent  $M_r$  of chicken ankyrin differs on different percentage SDS PAGE (data not shown), it is unclear at present what the differences in apparent  $M_r$  might signify. This awaits further investigation.

### *The Lower Steady-state Levels of Spectrin, Ankyrin, and Protein 4.1 in AEV-transformed Cells As Compared with Erythrocytes Are Due to Different Rates of Catabolism*

The lower steady-state levels of the peripheral components of the erythroid membrane skeleton observed in transformed cells as compared to mature erythrocytes could arise from lower rates of synthesis, different rates of catabolism, or a combination of the two. Short-term pulse labeling (10 min) was used to compare the amounts of labeled peripheral polypeptides synthesized in identical numbers of AEV-transformed cells, mitotic erythroblasts from 3.5–4-d embryos, and postmitotic immature erythrocytes from 10 and 14 d embryos (stages when these proteins are accumulating in the membrane skeleton on a per-cell basis (Blikstad et al., 1982; Moon and Lazarides, 1984; Woods and Lazarides, 1985). Surprisingly, the AEV cells synthesized these proteins on a per-cell per-unit time basis, at amounts that were equivalent to the amounts synthesized by mitotic erythroblasts and that were close to 10-fold greater than the amounts synthesized during a 10-min labeling period in postmitotic immature erythrocytes (Table II). Therefore, the low steady-state levels of these peripheral components in transformed cells compared to erythrocytes could not be accounted for by low rates of synthesis.

Kinetic pulse chase analysis revealed that in AEV cells, a portion of newly synthesized  $\alpha$ -spectrin,  $\beta$ -spectrin, ankyrin, and protein 4.1 polypeptides entered a Triton-soluble compartment and a portion became Triton-insoluble, i.e., cytoskeletal (Fig. 2). The excess hypotonic pools rapidly disappeared. The Triton-soluble pools of all of these proteins turned over in an apparently biphasic manner with an initial  $t_{1/2}$  of 45–60 min. Only a small portion of this loss could be accounted for by transfer into the insoluble pool. Thus it appears that most of the decline in this pool was due to rapid degradation. The insoluble pools also turned over, although with a slightly longer  $t_{1/2}$  than the Triton-soluble pools. In-



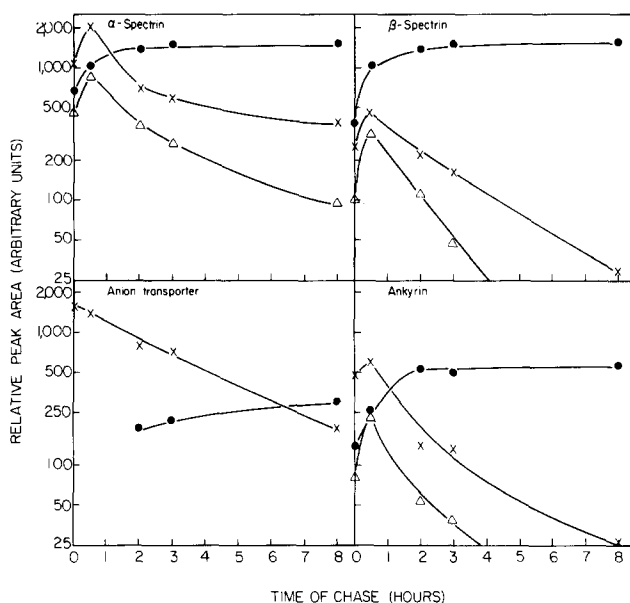
**Figure 2.** Kinetic analysis of the fate of newly synthesized polypeptides in AEV-transformed cells. AEV cells were pulsed for 10 min with [ $^{35}$ S]methionine and then divided into seven aliquots that were chased in a medium containing excess unlabeled methionine for up to 12 h. The aliquots of cells were fractionated into a hypotonically soluble (open triangle), Triton X-100-soluble (X) and -insoluble (solid circle) fraction. The labeled  $\alpha$ -spectrin,  $\beta$ -spectrin, ankyrin, and protein 4.1 were quantitated as described in Materials and Methods; values are expressed as the logarithm of relative peak areas and represent the mean value from four experiments. The data have not been corrected for any increase in cell numbers that occurred due to cell division during the chase period; rather they reflect the fate of the labeled polypeptides.

soluble  $\alpha$ -spectrin had a longer  $t_{1/2}$  than the other polypeptides of  $\sim 8$  h whereas insoluble  $\beta$ -spectrin, ankyrin, and protein 4.1 (all variants behaved in an identical fashion) all turned over with a  $t_{1/2}$  of  $\sim 4.5$  h (Fig. 2). Continuous pulse-labeling experiments indicated that the insoluble pools plateau around 4 h which confirms the fairly rapid turnover of these assembled proteins in AEV-transformed cells (data not shown). It should be noted that these half lives are considerably shorter than the doubling time (22–24 h) for these cells, so it seems unlikely that this instability could be linked to the fact that these cells are proliferating during this period of chase time. To distinguish between the possibility that the instability of the assembled pools of the spectrins, ankyrin, and protein 4.1 might be a consequence of the cells being a population of proliferative cells rather than being a feature unique to these cells and the stage of erythroid development that they represent, the fate of newly synthesized proteins in mitotic primitive erythroblasts from 3.5–4-d chick embryos was compared. Erythroblasts at this stage have been reported to have a doubling time of 17.5 h (Weintraub et al., 1971), i.e., similar to the doubling time of AEV-transformed erythroid cells. As seen in Fig. 3, the assembled pools in these cells appeared to be stable with no evidence of turnover during the chase time monitored (8 h).

**Table II.** The Relative Amounts of Labeled  $\alpha$ -Spectrin,  $\beta$ -Spectrin, and Ankyrin Synthesized during a 10-min Labeling Period

Protein	Relative amounts of the peripheral polypeptides synthesized		
	AEV cells	Erythroblasts from 3–4-d embryos	Erythroid cells from 10-d embryos
$\alpha$ -Spectrin	10.6	12.9	1
$\beta$ -Spectrin	9.4	10.1	1
Ankyrin	4.7	10.4	1

$3 \times 10^6$  cells were labeled for 10 min with [ $^{35}$ S]methionine and the amounts of labeled spectrins and ankyrin synthesized during this period were quantitated by immunoprecipitation followed by autoradiography. The data are expressed as amounts synthesized relative to the amounts synthesized in erythroid cells from 10-d embryos.



**Figure 3.** Kinetic analysis of the fate of newly synthesized polypeptides in mitotic primitive erythroblasts from 3–4-d chick embryos. Cells were pulse-labeled and fractionated exactly as described for AEV cells. The amounts of labeled  $\alpha$ -spectrin,  $\beta$ -spectrin, ankyrin, and the anion transporter in the hypotonically soluble (open triangle), Triton-soluble (X) and -insoluble (solid circle) fraction as a function of chase time are expressed in logarithmic form.

### ***AEV- and S13-transformed Erythroblasts Do Not Express Anion Transporter***

In contrast to the peripheral components of the membrane skeleton, immunoblotting with anti-anion transporter-specific antibodies failed to detect the presence of anion transporter in either AEV- or S13-transformed erythroid precursor cells. Using serial dilutions of the erythrocyte standard, we calculated that we should have been able to detect down to  $1/125$  the levels of anion transporter found in erythrocyte membrane skeletons. Since the stoichiometry of the different components in the mature membrane skeleton is known, it is possible to use the erythrocyte standard to calculate the stoichiometry that exists in the AEV-transformed cells. As shown in Table I, the  $\alpha$ -spectrin/ $\beta$ -spectrin/ankyrin/protein 4.1 ratio is  $\sim 5:1:0.5:0.5$  in AEV cells with similar values for undifferentiated S13 cells. In the mature erythrocyte membrane skeleton, the ratio of ankyrin to anion transporter is 1:1 with the skeletal-bound anion transporter accounting for only  $1/6$  the total amount of anion transporter (Bennett and Stenbuck, 1979b; Hargreaves et al., 1980; Branton et al., 1981; Nelson and Lazarides, 1984). Therefore, even if the transformed cells do contain trace levels of anion transporter, it is far below the levels relative to other skeletal components seen in erythrocytes and developing erythroid cells (see Fig. 3). Labeling experiments failed to detect the synthesis of the anion transporter in AEV-transformed cells. In contrast, proliferative erythroblasts synthesized high levels of anion transporter (Fig. 3). Although a large portion of the Triton-soluble pool turned over, a fraction of mature anion transporter assembled into the skeletal fraction in a stable manner. Northern analysis corroborated the lack of synthesis of anion transporter in AEV-transformed cells. As demonstrated in Fig. 4 an anion transporter-specific cDNA probe (Cox et al.,

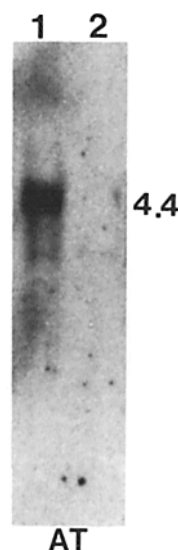
1985) failed to detect the presence of any anion transporter transcripts in cytoplasmic mRNA from AEV-transformed cells. In contrast a vimentin-specific cDNA probe detected vimentin-specific transcripts in the RNA from AEV-transformed erythroid cells (as a control, data not shown).

### ***AEV and S13 Cells Express Anion Transporter upon Spontaneous or Chemically Induced Differentiation***

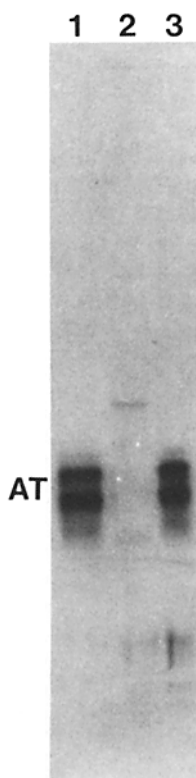
AEV-infected erythroid progenitor cells have a finite growth potential, generally extending over 3–4 mo. Thereafter, the cells appear to initiate differentiation spontaneously and then die. A similar sequence of events can be induced chemically by the addition of butyrate (1 mM) to the culture medium supplemented with 1 U/ml erythropoietin (Keane et al., 1979). Unlike murine erythroleukemia cells, AEV cells do not respond to DMSO (Keane et al., 1979; Graf and Beug, 1982). Initiation of differentiation, whether spontaneous or chemically induced, was associated with the appearance of the anion transporter  $\sim 3$  d after induction as detected by immunoblotting (Fig. 5) or immunofluorescence (data not shown). This was coupled with increased steady-state levels of  $\beta$ -spectrin, ankyrin, and protein 4.1 as judged by immunoblotting analysis (Table I). Before induction,  $<1\%$  of the AEV-transformed cells stained for hemoglobin with acid benzidine (Orkin et al., 1975). After 2 d in 1 mM butyrate,  $\sim 10\%$  of the cells stained positively with benzidine and after 3 d 24% stained strongly positive and 23% stained weakly positive for hemoglobin. The strongly positive cells were quite distinct in shape from the negative cells, being generally smaller in size and more elliptical in shape. A few possessed spikelet protruberances. The weakly positive cells were similar in shape to the avian reticulocytes described by Beug et al. (1982a, b). The same shaped cells that were strongly positive for hemoglobin were positive for anion transporter by immunofluorescence (data not shown).

### ***Discussion***

Understanding the mechanisms underlying differentiation, whereby the orderly activation or suppression in the expression of genes in response to environmental signals leads to



**Figure 4.** Northern analysis of cytoplasmic RNA from AEV cells compared with erythroblasts from 9-d chick embryos. Autoradiogram of a blot containing 1  $\mu$ g of poly(A)<sup>+</sup> RNA from chick erythroblasts (lane 1) and 10  $\mu$ g total cytoplasmic RNA from AEV cells (lane 2) that was hybridized to a  $^{32}$ P-labeled anion transporter cDNA probe.



**Figure 5.** The expression of the anion transporter upon induction of differentiation. Immunoblots of Triton-soluble (lane 1) and -insoluble fractions (lane 2) from  $3 \times 10^6$  AEV cells after spontaneous initiation of differentiation.  $3 \times 10^6$  mature chick erythrocyte ghosts (lane 3).

cellular diversity remains a crucial issue in biology. Erythropoiesis is initiated as the replicative progeny of pluripotent stem cells becomes restricted in its developmental potential and committed to the erythroid lineage. After a minimum of two replicative stages, BFU-E and CFU-E, where the erythroid progenitor cells become increasingly more responsive to erythropoietic inducers, the cells begin to differentiate terminally as evidenced by the appearance of hemoglobin (Metcalf and Moore, 1971; Till and McCulloch, 1980; Weiss, 1984). Unlike other cell lineages, e.g., muscle, where terminal differentiation is initiated as the cells become postmitotic, in erythroid cells terminal differentiation as defined by the appearance of hemoglobin, is initiated while erythroblasts are still mitotic. These cells undergo a finite number of cell divisions which are accompanied by a sequence of maturation steps leading to postmitosis and the ultimate erythrocyte phenotype (Till and McCulloch, 1980; Weiss, 1984). In avian species part of this maturation process entails the elaboration of three interlinked skeletal domains that are responsible for the morphology of the mature erythrocyte; a spectrin-actin based membrane skeleton (this domain is common to both avian and mammalian erythrocytes) (Branton et al., 1981; Lazarides, 1985; Marchesi, 1985); a system of vimentin-based intermediate filaments that appear to interlink the nucleus with the plasma membrane (Harris and Brown, 1971; Haggis and Bond, 1979; Granger and Lazarides, 1982), and a marginal band of microtubules, laterally disposed to the intermediate filament network (Benhke, 1970; Swan and Solomon, 1984; Murphy et al., 1986). The elaboration of one of these domains, the intermediate filament network has been shown to occur as the erythroid cells become postmitotic (Capetanaki et al., 1983). Basal levels of vimentin mRNA and vimentin filaments are present throughout the mitotic maturation phase of erythroblasts but they are aug-

mented when the cells become postmitotic. The timing of the initiation of expression and the control of assembly of the components of the membrane skeleton during the various phases of erythroid terminal differentiation are less well established.

Studies with early proliferative or postmitotic erythroblasts of both the primitive and definitive lineage from chick embryos have revealed that these cells are expressing and assembling all the major components of the membrane skeleton simultaneously (e.g.,  $\alpha$ -spectrin,  $\beta$ -spectrin, ankyrin, anion transporter, and protein 4.1) (Chan, 1977; Weise and Chan, 1978; Blikstad et al., 1983; Moon and Lazarides, 1984; Woods and Lazarides, 1985; Staufenbiel and Lazarides, 1986). The first indication of asynchrony in the expression of these proteins appears late in terminal differentiation in the case of protein 4.1 whose synthesis is maintained beyond that of the other components during the final maturation stages of postmitotic primitive and definitive erythrocytes (Staufenbiel and Lazarides, 1986). The situation with mammalian reticulocytes is less clear. In vivo labeling of mice and anemic rabbits indicates that synthesis of membrane skeletal components occurs late in development before the reticulocyte stage but an asynchrony in the termination of synthesis is also observed. In one case, synthesis of band three (anion transporter) continued beyond that of the peripheral components (Chang et al., 1976), whereas in another case the synthesis of the spectrins, ankyrin, and band three terminated simultaneously with the synthesis of band 4 and 5 (actin) continuing into the reticulocyte stage (Koch et al., 1975). Circulating reticulocytes in anemic sph/sph mice which carry a lesion in  $\alpha$ -spectrin (Bodine et al., 1984), have been shown to synthesize all the other major components of the membrane skeleton. These studies suggest that the expression and assembly of the membrane skeleton is a late event of the erythropoietic pathway but do not address the issue of at what stage of erythropoiesis these proteins begin to be expressed or whether there is any asynchrony in the initiation of expression of the different components. Friend murine erythroleukemia (MEL) cells have provided one model system to study this issue since they represent erythroid cells that are transformed at an early proerythroblast stage but can be induced chemically to undergo terminal differentiation. Before induction, MEL cells express only the more generalized form of spectrin, namely  $\alpha\gamma$ -spectrin and very low levels of erythroid-specific spectrin,  $\alpha\beta$ -spectrin, but once chemically induced to initiate differentiation, the cells begin to express elevated levels of erythroid spectrin (Eisen et al., 1977; Glenney and Glenney, 1984), band three (Sabban et al., 1982), and glycophorin (Ikawa et al., 1973) concomitantly with hemaglobin, thus suggesting that synthesis of at least these membrane skeletal components begins simultaneously upon initiation of terminal differentiation. However, MEL cells induced to differentiate in vitro differ from normal erythroid progenitor cells in being erythropoietin independent. Furthermore, they do not go through the normal sequence of erythroblast stages (Marks and Rifkind, 1978). Therefore, it is unclear how closely events observed in this model system mimic normal erythropoiesis.

Chicken erythroid progenitor cells transformed with the avian erythroblastosis and S13 viruses differ in these regards since when induced to differentiate they do so in an eryth-



ropoietin-dependent manner giving rise, by morphological criteria, to fully differentiated erythrocytes through the normal early erythroblast and polychromatophilic erythroblast (the avian equivalent of reticulocyte) stages (Graf and Beug, 1978; Graf et al., 1978; Beug et al., 1982a, b). Furthermore, the evidence suggests that AEV cells are transformed cells that are blocked in their differentiation pathway at the CFU-E stage rather than being cells that have somehow dedifferentiated as a consequence of transformation (Graf and Beug, 1978; Gazzolo et al., 1980a, b; Samarut and Gazzolo, 1982).

Previous studies have failed to detect the presence of spectrin by immunofluorescence in AEV-transformed erythroblasts (Beug and Hayman, 1984). The observations reported here indicate that not only do AEV- and S13-transformed cells express  $\alpha$ -spectrin but also erythroid-specific peripheral components, namely  $\beta$ -spectrin, ankyrin, and protein 4.1. However, they do not appear to express anion transporter, the protein that is thought to serve as one of the major transmembrane receptors for the erythroid skeleton. This lack of anion transporter protein at steady state appears to be regulated at the level of mRNA as evidenced by the lack of synthesis of this protein and the lack of any detectable anion transporter mRNA. At steady state, both types of transformed cells contain greatly reduced levels of  $\alpha$ -spectrin,  $\beta$ -spectrin, ankyrin, and protein 4.1 than found in mature erythrocyte membrane skeletons. Although the ratios of erythroid-specific components found in both AEV- and S13-transformed cells are similar to the ratios found in proliferative erythroblasts (Staufenbiel and Lazarides, 1986), the ratio of  $\alpha$ -spectrin to  $\beta$ -spectrin is 5:1, which differs from the 1:1 stoichiometry seen in developing and mature erythrocytes. This we presume to reflect the concurrent expression of  $\alpha\gamma$ - and  $\alpha\beta$ -spectrin, a situation that is analogous to that observed in MEL cells (Glenney and Glenney, 1984). If this is indeed the case, then it would explain the slower rate of turnover of the insoluble  $\alpha$ -spectrin observed in the transformed cells compared to the erythroid-specific components, since the turnover rate would reflect the average value of two  $\alpha$ -spectrin-containing pools. Unlike mammals, avian species possess a single copy gene for  $\alpha$ -spectrin (Moon et al., 1985) and the same gene product is found in both erythroid ( $\alpha\beta$ ) and nonerythroid ( $\alpha\gamma$ ) spectrin. In the absence of a probe for  $\gamma$ -spectrin we were unable to distinguish between the two  $\alpha$ -spectrin-containing forms.

The induction of expression of proteins that are characteristic of a terminally differentiated cell lineage is typically not an absolute one; rather a low level of expression is seen before terminal differentiation but this is augmented after initiation of terminal differentiation leading to an increase in their steady-state levels. For example, this is seen in the case for globin (Marks and Rifkind, 1978; Keane et al., 1979; Weintraub et al., 1982) and vimentin (Capetanaki et al., 1983) in developing erythroblasts. Therefore an obvious explanation of the much lower steady-state levels of the peripheral membrane skeletal components in AEV- and S13-transformed cells would be a lower expression of these proteins compared to maturing erythroblasts. Surprisingly, this was found not to be the case. Their levels of synthesis in AEV cells were equivalent to the levels observed in early proliferative primitive erythroblasts. They were also several-fold higher than the levels observed in postmitotic immature erythrocytes at the stage where the membrane skeletal compo-

nents are accumulating and the steady-state levels are 20–50-fold higher. However, unlike proliferative primitive erythroblasts, AEV cells exhibited a high rate of turnover of assembled  $\alpha$ -spectrin,  $\beta$ -spectrin, ankyrin, and protein 4.1. Thus it appears that the low steady-state levels of these polypeptides in these cells compared to erythroblasts are due to differences in rates of catabolism rather than differences in rates of synthesis. The fact that these molecules appear to assemble in a stable fashion in proliferative primitive erythroblasts isolated from 3–4-d embryos which have been reported to have a doubling time of 17.5 h (Weintraub et al., 1971) which is similar to the generation time of AEV-transformed erythroid cells (22–24 h) eliminated the possibility that this marked difference in stability was due to the fact that AEV cells are actively proliferating. Rather, this instability appears to be a unique feature of the AEV-transformed cells and the stage of development that they represent.

AEV transformation of erythroid progenitor cells is finite, generally lasting between 40 and 60 generation times before the cells lose their transformed character, begin to differentiate and then after 3–5 d in culture they die. When cells were sampled before this time, it was found that concomitantly with this spontaneous initiation of differentiation they began to express anion transporter. Furthermore, this was associated with an increase in the steady-state levels of the peripheral components. A more careful analysis indicated that the cells began to accumulate anion transporter 3–4 d before cessation of growth and cell death. It is interesting to note that normal CFU-E cells go through 3–5 divisions before developing into erythrocytes (Till and McCulloch, 1980). AEV cells can also be induced to differentiate by addition of butyrate together with erythropoietin to the cultures. Again chemically induced AEV cells accumulated anion transporter and concurrently the steady-state levels of the erythroid-specific peripheral components increased. Thus, assuming that the pattern of differentiation induced by butyrate does not differ from the natural sequence of events, these results provide additional evidence that an asynchrony exists in the expression of the peripheral components and the anion transporter during erythropoiesis.

Overall the results reported here indicate that the differences that exist in the steady-state levels of the peripheral components of the erythroid membrane skeleton between the AEV-transformed erythroid cells and erythroid cells of later stages of development are determined by differences in susceptibility to catabolism. They further point to a key role for the anion transporter in controlling the steady-state levels of the peripheral components during terminal differentiation by stabilizing assembled molecules from catabolism. However, these polypeptides do appear to be competent to assemble onto the plasma membrane of transformed cells in the absence of anion transporter. By immunofluorescence the spectrins, ankyrin, and protein 4.1 all localize under the plasma membrane. In addition, the solubility properties, steady-state ratios, and the identical turnover rates for all the erythroid-specific polypeptides are consistent with the conclusion that they are forming a complex at the membrane but are inefficiently cross-linked into a skeletal array. Therefore, these results further suggest that there must exist additional membrane-binding sites other than the anion transporter for the  $\alpha\beta$ -spectrin/ankyrin/protein 4.1 complex, albeit sites



that are less efficient in stabilizing the assembled polypeptides than the anion transporter. One possibility is that these cells are expressing glycophorin, a transmembrane protein of the mature erythrocyte that has been purported to bind protein 4.1 in human erythrocytes. An avian equivalent of glycophorin has not been identified to date, and so this possibility remains to be tested.

The issue to be addressed is whether the asynchrony between the expression of the anion transporter and the peripheral constituents of the membrane skeleton reflects the normal sequence of events during erythropoiesis, i.e., the normal phenotype of the CFU-E cells, or whether it represents an anomalous pattern of expression induced by transformation. Either the site of insertion of the proviruses into the chicken genome or the activity of their respective oncogene products could be envisaged to disrupt the normal sequence of expression of erythroid-specific proteins. Since it is known that retroviral DNA can integrate at many sites in cellular DNA, apparently at random (Hughes et al., 1978), any transformation-specific effect on the expression of erythroid-specific proteins is most likely to be caused by the activity of the oncogene products of AEV and SI3. The expression of the major oncogene of AEV, v-erb B, has been shown to be required for transformation (Beug et al., 1982a) and the expression of the gpl55 protein encoded by the oncogene v-sea of the SI3 genome has been correlated with transformation (Benedict et al., 1985; Beug et al., 1985). It has recently been demonstrated that gpl55 is cleaved posttranslationally giving rise to two glycoproteins, gp85 and gp70. The latter has been shown to possess tyrosine protein kinase activity and so in this regard bears a functional similarity to v-erb B gene product (Hayman et al., 1985). However, there is no structural homology between v-erb B and v-sea (Benedict et al., 1985). Until the protein substrates for these two types of tyrosine protein kinases in the transformed erythroleukemic cells are identified, the possibility exists that this activity may somehow be anomalously affecting the expression or the stability of either the peripheral components or the anion transporter. Alternatively, it could be argued that these oncogenic tyrosine protein kinase activities could just be attenuating the normal growth signals of erythroid progenitor cells at this stage of differentiation. Thus at present, it appears reasonable to conclude that the results reported here may reflect the normal state of affairs of the stage of erythroid differentiation these cells are blocked at by these two classes of virus.

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